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Cloning and analysis of promoter elements of a Ser/Thr protein kinase gene homologue from *Piper colubrinum* Link.

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Serine/Threionine (Ser/Thr) protein kinases are multifarious phosphorylation enzymes which also play a central role in signalling events following pathogen recognition in plants. The present study describes cloning and characterisation of upstream promoter region of a Ser/Thr protein kinase (STPK) gene from *Piper colubrinum* (*PcSTPK*), a wild species highly resistant to fungal pathogens in the *Piper* germplasm. The gene was found to be pathogen responsive when challenged with oomycete pathogen – *Phytophthora capsici*. Transcript abundance of *PcSTPK* was determined by quantitative real time-polymerase chain reaction (qRT-PCR) which demonstrated maximum transcript accumulation of *PcSTPK* in inflorescence, followed by leaf, stem and root tissues. Inoculation of leaf tissues with the oomycete pathogen *Phytophthora capsici*, also induced significant transcript accumulation of *PcSTPK* in the plant. Genome walking methodology was adopted to clone upstream promoter elements of *PcSTPK*. *In silico* analysis revealed the presence of regulatory elements for light responsiveness, meristem and endosperm expression in addition to various hormone responsive elements. Our results suggest that *PcSTPK* along with its *cis*-regulatory elements has a role in modulation of plant stress response.

Keywords: Protein kinases; receptor like kinases; promoter; disease resistance

Introduction

Protein kinases are crucial for the recognition of pathogens and defense responses in plants[1]. In most cases, defense signal perception is accompanied by protein phosphorylation, which activates transcription factors to induce pathogenesis-related (*PR*) genes, leading to local and systemic resistance responses, such as callose deposition and the hypersensitive response[2-5]. Protein kinases are considered as important nodes of cell machinery where they process input signals from internal/external environment and trigger downstream signalling which eventually leads to a distinct cellular response[6-8]. Of the various types of protein receptor kinases in plants, the receptor-like kinases (RLKs) contain C-terminal intracellular serine/threonine (Ser/Thr) kinase domains and/or versatile N-terminal extracellular domains. Their overall structure suggests a role of the extracellular domain in the perception of an extracellular ligand and signal transduction through the intracellular kinase domain. This family includes genes regulating developmental processes and plant defense, such as *FLS2* and *Xa21*[9]. Some protein kinases in this family lack extracellular domains and are localized to intracellular environment and they are now termed as receptor like cytoplasmic kinases (RLCKs). It is important to understand how kinase-mediated signalling cascades are coordinated in a defense response since many protein kinases are involved at various levels of such interactions - from the early detection of pathogen to mounting a visible physiological response in tissues[10-12]. Protein kinases are potential targets for genetic manipulation for transgenic solution for crop improvement as they regulate cascades of molecular events in plant stress reactions[13-18].

Wild *Piper* species, *P. colubrinum* Link., is highly resistant to many of the pathogens attacking cultivated black pepper (*P. nigrum* L), including *P. capsici* which causes the most devastating 'foot rot' disease. The plant is considered to be a rich repository of defense genes and hence has application in molecular breeding of black pepper. Major biotechnological interventions are needed for crop improvement of *P. nigrum* as many of the horticulture practices and conventional breeding strategies have met with limited success[19-20] till date. A salicylic acid induced

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subtracted library was generated in *P. colubrinum* in our laboratory for the discovery of candidate genes and regulatory elements as an essential step towards genetic improvement of cultivated pepper[21]. The Ser/Thr protein kinase gene (*PcSTPK*) was identified as a differentially over expressed gene (EB104037) in the subtracted library generated earlier in our laboratory. Transcript level expression analysis indicated that the gene is overexpressed in this plant in response to the 'foot rot' disease pathogen, *P. capsici*. Signalling molecules like ethylene and salicylic acid also induces the gene expression. *PcSTPK* when subjected to RNAi-mediated transient gene silencing, caused a co-ordinated transcript down regulation of a set of downstream defense-related genes in *P. colubrinum*[22]. Further, it was also shown that silencing of *PcSTPK* gene using virus induced gene silencing (VIGS) methodology led to increased susceptibility in *P. colubrinum* against oomycete pathogen *Phytopthora capsici*[23]. These data suggest a possible major role for *PcSTPK* in regulating the expression of defense genes. The present study describes the cloning and characterisation of *PcSTPK* upstream promoter fragment from wild *P. colubrinum*, which is an essential step for further analyses on its defense functional role and mode of regulation. Our data indicate the possible involvement of a receptor like cytoplasmic kinases (RLCK) in *Piper* defense to fungal pathogens, which needs to be validated further for its implications in crop improvement programmes.

Materials and Methods

Plant Material and Treatment

Green house grown healthy *P. colubrinum* plants maintained under uniform growth conditions were used for the present study. Fresh tissues including leaves, stem, roots and spike (inflorescence) were collected for cloning and expression analysis. One year old stem cuttings of *P. colubrinum* were used for inoculation studies with pathogen (*P. capsici*). For pathogen inoculation, discs of fungal mycelium cultured on potato dextrose agar were placed on the surface of young leaves. Pathogen treated leaves were covered with polythene bags and samples were taken periodically for expression analysis. Polythene bag covered, untreated plants served as control.

RNA and DNA Isolation

Total RNA was isolated from leaf, stem, root and spike tissues by trizol method (Invitrogen, Carlsbad, CA, USA). Approximately 1 µg of DNase (Sigma, USA) treated RNA was used to prepare cDNA using MMLV-RT following manufacturer's protocol (Promega, Madison, WI, USA). The isolated RNA sample was analysed on 2% ethidium bromide (EtBr) agarose gel and quantified using Nanodrop (Thermo Fischer Scientific, USA). Genomic DNA was isolated from young leaves using Genelute plant genomic DNA isolation kit (Sigma, USA), following manufacturer's instruction. The quality of genomic DNA was analysed in 0.8% EtBr-agarose gel and quantified using Nanodrop (Thermo Fischer Scientific, USA).

Expression Analysis of *PcSTPK*

Complementary DNAs (cDNAs) were synthesized from total RNA collected from different tissues using MMLV reverse transcriptase (Promega, Madison,WI, USA). Gene specific primers RTSTPK1 and RTSTPK2 (Table 1) designed based on the sequences of *PcSTPK* (JQ398495) were used for real-time PCR analysis. The reaction was set up to a final volume of 20 µL containing 10 µL SYBR green PCR reagent

Table 1 — Primers used in the present study. The table shows the sequences of the Primers used in the present study. The primers GW1, GW2 and M13 F were used for sequencing.

Primers used in the present study			
Name	Sequence	Binding site	Accession number
GSP1	5'-CCTGAACAAGCCGGATGCTGAAAAC-3'	202-226	JQ398495.1
GSP2	5'-GGAGGCGATTGAAATGAACCCAGAG-3'	83-107	JQ398495.1
RTSTPK1	5'□ TCAGCAAAATCAGGTCTTCCAT□3'	1511-1533	JQ398495.1
RTSTPK2	5'□ GTAGGTGTTGTTCAAAAGGGTC□3'	1414-1435	JQ398495.1
RTACTF	5'-ACATCCGCTGGAAGGTGC-3'		
RTACTR	5'-TCTGTATGGTAACATTGTGCTC-3'		
AP1	5'-GTAATACGACTCACTATAGGGC-3'		
AP2	5'-ACTATAGGGCAGCGTGGT-3'		
GW1	5' GCTACTGCCCAGACTTGCATT 3'		
GW2	5' CATGGATCACTCAACATTTC 3'		
M 13F	5' GCTACTGCCCAGACTTGCATT-3'		

(Applied Biosystems, CA, USA), Two µL of diluted cDNA and 300 nM each of the designed primers and the conditions were: 50°C for 2 min initially followed by 95°C for 10 min and 40 cycles of 95°C for 15 s and 60°C for 1 min in a real time PCR machine (ABI 7500; Applied Biosystems, CA, USA). The housekeeping gene *β-actin* cloned from *P. colubrinum* was used for normalisation and the relative expression level of the genes was determined by comparing the *Ct* values using the SDS software (Applied Biosystems, CA, USA). In each experiment, at a time, two biological replicates for an event were used. Three technical replicates of each biological replicate were used for the analysis. The results were subjected to Student's t-test to validate the extent of transcript accumulation of *PcSTPK* .

Cloning of *PcSTPK* Promoter by Genome Walking

Genome walker libraries were prepared from *P. colubrinum* using the GenomeWalker™ universal kit (Clontech, USA) following manufacturer's protocol. Genomic DNA isolated from fresh leaves was digested with four blunt-ended enzymes, *Dra*I, *Eco*RV, *Pvu*II and *Stu*I. The resulting genomic fragments were then ligated to genome walker adaptors provided in the kit, to generate four Genome walker libraries. Gene specific primers GSP1 and GSP2 designed based on the sequence of *PcSTPK* (JQ398495) was used for two-round of PCR reactions (primary and secondary) to amplify the promoter sequence. For the first round PCR reaction, the primers GSP1 and AP1 (Primer provided with the kit) were used, the second round PCR (nested PCR) reaction was setup using the primers GSP2 and AP2 provided with the kit. Primary PCR was set up for each separate library in 50 µL reactions, containing 40 µL sterile water, 5 µL 10X advantage 2 PCR buffer, 1 µL dNTPs (10 mM), 1 µL each of AP1 and GSP1 primers (10 µM), 1 µL advantage 2 polymerase mix and 1 µL each of the respective genome walker (GW) library. PCR cycling conditions were set to seven cycles of 94°C for 25s and 68°C for 3 min followed by 32 cycles of 94°C for 25s and 65°C for 3 min and a final extension at 67°C for 7 min. PCR products (5 µL each) were checked on a 1.2% agarose/EtBr gel. For secondary PCR, an aliquot of primary PCR product was diluted fifty times and 1 µL of this was used as template. PCR reaction conditions were same as that of primary PCR except for the use of GSP2 and AP2 as primers. The longest fragment obtained was selected and cloned in gateway compatible entry vector pCR8GWTOPO (Invitrogen, CA, USA). The ligated products were introduced into *E. coli* strain DH5α and transformed cells were selected on spectinomycin (50 µg/mL) supplemented Luria Bertani (LB) medium. Plasmids harbouring the secondary PCR products were isolated and sequenced ABI Prism Automated Sequencer (Applied Biosystems, Foster City, CA, USA). The primer sequences are provided in Table 1.

***In silico* Analysis of *PcSTPK* Promoter Sequences**

The location and distribution of *cis*-regulatory sequence elements in the *PcSTPK* promoter were analyzed by signal scan search using databases like PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) or PLACE (http://www.dna.affrc.go.jp/PLACE/). The closest homologues were identified by a homology based search in the PlantCARE database.

Results

Expression Analysis of *PcSTPK*

Quantitative real time PCR (qRT-PCR) analysis was conducted to study the tissue specific expression pattern of *PcSTPK* in the leaf, stem, root and spike (Fig. 1a). The results indicate that *PcSTPK* is expressed in all tissues examined with a significantly higher expression level in spike (inflorescence tissue) ($p < 0.05$, $n = 6$). Among leaf and stem tissues *PcSTPK* expression showed no significant variation. Expression of *PcSTPK* was also analysed for its response towards the pathogen-*Phytophthora capsici*. It is noteworthy that treatment with *P. capsici*, caused rapid and significantly higher expression of *PcSTPK* **(**Fig. 1b**),** which persisted for 48 hours.

***PcSTPK* Promoter Cloning and *In Silico* Analysis**

Genome walking method produced amplicons of different lengths from *Dra*I and *Eco*RV library (Fig. 2a & b). The longest PCR product was obtained from *Eco*RV genome walker library which was about 4 kb. The sequence of the proximal *PcSTPK* promoter (2874 bases) as well as the position of various regulatory sequences is presented as Figure 3. Online available promoter/*cis* element databases PlantCARE and PLACE were used to identify *cis*-regulatory elements in the *PcSTPK* promoter fragment. A putative TATA-box and CAAT-box was detected at positions -179, and -277 respectively, upstream of the translation start site (Fig. 3). Potential *cis*-regulatory elements were also identified upstream to the basal

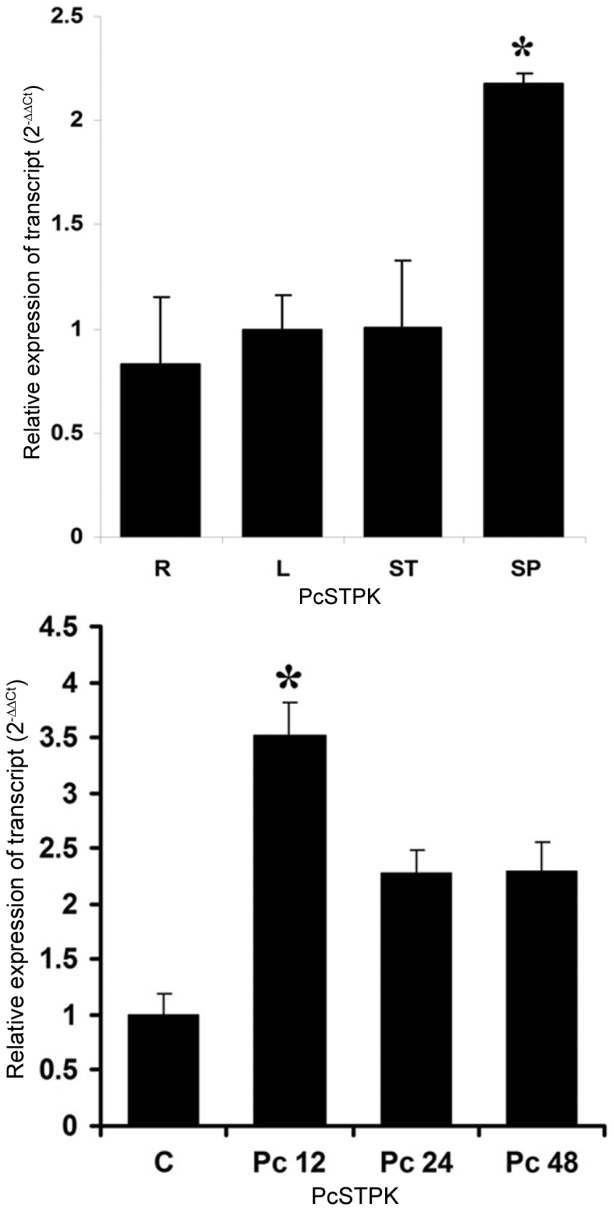

Fig. 1 — Expression analysis of *PcSTPK* (a) Tissue specific expression analysis of *PcSTPK*. L-Leaf, R-Root, ST-Stem, SP-Spike, (b) Expression analysis of *PcSTPK* in leaves treated with *Phytophthora capsici*. *PcSTPK* exhibits rapid transcript accumulation in the presence of the pathogen and it remains at elevated levels after the initial sudden response. The gene expression levels are shown as relative fold change employing the $2^{-\Delta\Delta Ct}$ method. * - significance as shown by Student's t-test.

core promoter region on the sequence. Stress-regulated elements, such as Box-W4 (light responsive element), Box II (light responsive element), MNF1 (light responsive element), CAT box (meristem expression), MBS (MYB binding site), Skn-1 (*cis*-acting element involved in endosperm expression), GCN4 (endosperm expression), HD-Zip1 (*cis*-element for leaf morphology), HSE (heat stress element) and

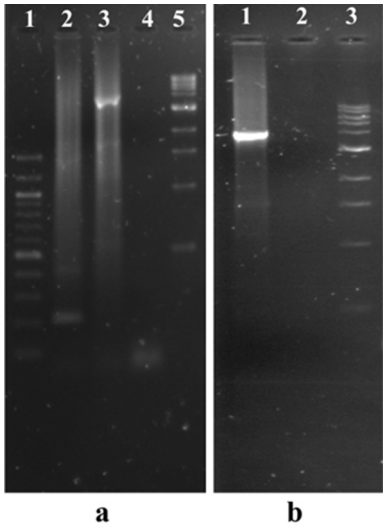

Fig. 2 — Cloning of *PcSTPK* promoter elements by genome walking: a) Primary PCR, Lane 1-100 bp ladder (NEB), 2-Primary amplification from *Dra*I genome walker library, 3-Primary amplification from *Eco*RV genome walker library, 4-negative control, 5-1 Kb ladder (NEB), b) Secondary PCR Lane 1- Secondary amplification of primary PCR product from *Eco*RV genome walker library. Lane 2-negative control, Lane 3-1 Kb ladder

cis-elements for circadian control were identified (Fig. 3). In addition, putative hormone-responsive elements including TCA-element (salicylic acid responsive element), MeJA (jasmonic acid responsive element), auxin RR (auxin responsive element) were also found.

Discussion

Protein kinases (Ser/Thr protein kinases, EC 2.7.11.1) are host immune receptors which are known to play a role in ETI through phosphorylation of the -OH group of serine or threonine residues leading to a functional change of the target protein. Protein phosphorylation has been identified as one of the most important events in disease resistance and developmental pathways[1,2,24-26]. Many protein kinase homologues have been identified in plant-microbe interactions[3,26-29]. Some of them directly interact with pathogen-derived avirulent (*Avr*) gene products and elicit downstream signalling resulting in transcription factor activation, systemic responses etc[2-12]. Present day plant stress biology focus on protein kinase-based exploratory studies that will help to characterise the signalling cascade and its components[27-34]. The future challenge of such studies will be the biochemical and genetic characterisation of regulators, phosphorylation targets and other interacting partners involved in a signaling event[35]. Sequence analysis of *PcSTPK*

Fig. 3 — Sequence of *PcSTPK* promoter elements: *PcSTPK* promoter sequence upstream of ATG (translation start codon) is shown (2874 bp). Numbering is from the first base of translation start site (+1). Putative promoter elements like TATA boxes, CAAT boxes are italicized and shown in red colour and highlighted. The translation start site ATG is italicized, and shown in red colour. Following are the important promoter elements identified in the cloned sequence : TCAGATGAG (-2758)TCA element (SA responsiveness), GTCAT- (-2637, -2493, -2082, -1191, -1164) Skn-1 motif (endosperm expression), GCGACGCG (-2443) ABRE, AACCTAA (-2340) MRE ,MYB binding site /light responsive element), GGTCCAT (-2026) AuxinRR, GAAGAATAAA - (-2009) SA responsiveness, AGAGAAGAAAA (-1935) TCA SA responsiveness, CGTCAACTCAAT - (-1687)MeJA response, CAAAAGCCACT (-1469) CAT box meristem expression, AAAAAAT (-1366) HSE heat stress, TGACG (-1313) MeJ, GCATTC (-1302) CATT motif (light responsive), CACGTG (-971) ABRE, ATTAA (-897, -890, -699) Box-4 , AACCTAA (-618) MRE MYB binding site, GCATTC (-605) CATT motif.

showed its location in cytoplasm and predicted as a member of the receptor like protein class, indicated by phylogenetic analysis[23].

Present study describes successful cloning of promoter element of a Ser/Thr protein kinase gene promoter from *P. colubrinum*. Plant resistance to pathogens is controlled by a combination of pathogen defense response pathways that are triggered depending on the nature of the pathogen and its modes of pathogenesis[1,9,11,12,25,32,33]. Receptor like protein kinases (RLPs) represent a big family of protein kinases, of which majority are transmembrane receptor kinases and rest are receptor like cytoplasmic kinases (RLCK) or receptor like proteins (RLP). RLCKs like M-locus protein kinases and *Pto* kinases do not possess extracellular domains. Recent studies have highlighted the importance of receptor like cytoplasmic kinase in mediating pathogen triggered immunity as well as effector triggered immunity[1,12,33,34].

Recent reports on the use of protein kinase genes focus on their potential use in crop improvement programme by transgenic approach[26,33,35]. However, the exact function of a large number of these protein kinases is quite unknown. RLCKs identified in *Oryza*, for instance, are yet to be functionally characterised[34-35]. Detailed genetic, molecular and biochemical analyses are required as these emerging molecules often have integrated roles which control different cellular pathways in response to specific stimuli[12,34-35]. It is worth mentioning at this point that a transient endogenous gene silencing of *PcSTPK* conducted earlier in our laboratory, effected a correlated downregulation of many transcripts which were differentially expressed in *P. colubrinum* in response to defense signal molecule salicylic acid[22]. In our present study, *in silico* analysis of the upstream promoter elements revealed some of the key regulatory elements that could be involved in the expression of *PcSTPK* under various conditions. It will help further functional characterisation studies of *PcSTPK* with respect to defense signalling in the resistant *Piper* species. It is important to identify/understand the functions of *cis*-elements associated with various classes of receptor molecules as it will help to elucidate the mechanisms of intracellular or extracellular signal perception. Upon analysis it was apparent that the proximal part of promoter harbors salicyclic acid and jasmonate responding elements along with elements responsible for basal level expression. Functional analysis of cloned promoter harboring pathogen responsive elements will help us to better understand behaviour of the gene in the context of pathogenesis[36-37]. Pathogen responsive promoter elements either from the wild or cultivated germplasm will be of great use in crop genetic engineering[38-40]. There are several instances where isolated promoter elements are further used for biotechnological crop improvement[40-52]. In many crop breeding strategies, for disease and abiotic stress tolerance, novel regulatory elements capable of fine tuning of spatio-temporal regulation of genes are needed[53-55]. Such tissue specific or inducible promoters can greatly reduce the pleiotropic effect of constitutive expression of gene products and also investment of metabolic energy at unnecessary sites or period of time[39,51,53]. Further, it can also be seen that analysis of promoter sequences might shed light on the tissue specific regulation involved in the biosynthetic pathways[46,49].

Wild species have been widely used as genetic resources for introgression of useful traits into cultivated species by wide hybridization[55]. Cloning of genes and regulatory elements from the wild relatives and use of these genes in transgenic studies is an efficient way for modern genetic improvement. In a recent report, Cao *et al* [24] demonstrated that a putative serine and threonine protein kinase *Stpk-V* cloned from a wild relative species of wheat confers strong durable resistance in cultivated wheat towards powdery mildew[38]. But, in crops like *P. nigrum*, limited germplasm resources and incompatibility in crosses between distantly related wild and cultivated species pose a significant hurdle for conventional breeding. The present report of identification of a putative defense related gene and its promoter from resistant *Piper* species will have significant potential in *P. nigrum* genetic engineering in future. Cloning of genes and its regulatory elements from wild relatives of black pepper will extend the current knowledge base in *Piper* genomics as an aid for future in-depth high throughput approaches for molecular breeding in cultivated *P. nigrum*.

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Reference

1 Afzal A J, Wood A J & Lightfoot D A, Plant receptor-like serine threonine kinases: Roles in signaling and plant defense, *Mol Plant Microbe Int*, 21 (2008) 507-517.

2 Romeis T, Protein kinases in the plant defence response, *Curr Opin Plant Biol*, 4 (2001) 407-414.

3 Swiderski M R & Innes R W, The *Arabidopsis* PBS1 resistance gene encodes a member of a novel protein kinase subfamily, *Plant J*, 26 (2001) 101-112.

4 Asai T, Tena G, Plonikova J, Willmann M, Chiu WL *et al*, MAP kinase signalling cascade in *Arabidopsis* innate immunity, *Nature*, 415 (2002) 977-983.

5 Zhang S & Liu Y, Activation of salicylic acid - induced protein kinase, a mitogen-activated protein kinase, induces multiple defense responses in tobacco, *The Plant Cell*, 13 (2001) 1877-1889.

6 Hardie DG, Plant protein serine/threonine kinases: Classification and functions, *Ann Rev Plant Physiol Plant Mol Biol*, 50 (1999) 97-131.

7 Zhu J K, Salt and drought stress signal transduction in plants, *Ann Rev Plant Biol*, 53 (2002) 247-273.

8 Chevalier D & Walker J C, Functional genomics of protein kinases in plants, *Brief Funct Gen & Prot*, 3 (2005) 362-371.

9 Tichtinsky G, Vanoosthuyse V, Cock J M & Gaude T, Making inroads into plant receptor kinase signalling pathways, *Trends in Plant Sci*, 8 (2003) 231-237.

10 Mur L A J, Kenton P, Lloyd A J, Ougham H & Prats E, The hypersensitive response; The centenary is upon us but how much do we know?, *J Exp Bot*, 59 (2008) 501-520.

11 Ludwig A A, Saitoh H, Felix G, Freymark G, Miersch O *et al*, Ethylene-mediated cross-talk between calcium-dependent protein kinase and MAPK signaling controls stress responses in plants, *Proc Natl Acad Sci USA*, 102 (2005) 10736-10741.

12 Zhang J, Li W, Xiang T, Liu Z, Laluk K *et al*, Receptor-like cytoplasmic kinases integrate signaling from multiple plant immune receptors and are targeted by a *Pseudomonas syringae* effector, *Cell Host & Microbe*, 7 (2010) 290-301.

13 Mysore K S, D'Ascenzo M D, He X & Martin G B, Overexpression of the disease resistance gene *Pto* in tomato induces gene expression changes similar to immune responses in human and fruitfly, *Plant Physiol*, 132 (2003) 1901-1912.

14 Yamamizo C, Kuchimura K, Kobayashi A, Katou S, Kawakita K *et al*, Rewiring mitogen-activated protein kinase cascade by positive feedback confers potato blight resistance, *Plant Physiol*, 140 (2006) 681-692.

15 Kobayashi M, Ohura I, Kawakita K, Yokota N, Fujiwara M *et al*, Calcium-dependent protein kinases regulate the production of reactive oxygen species by potato NADPH oxidase, *The Plant Cell*, 19 (2007) 1065-1080.

16 Coello P, Hay S J & Halford N G, The sucrose non-fermenting-1-related (SnRK) family of protein kinases: Potential for manipulation to improve stress tolerance and increase yield, *J Exp Bot*, 62 (2011) 883-893.

17 Zhang H, Mao X, Wang C & Jing R, Overexpression of a common wheat gene TaSnRK2.8 enhances tolerance to drought, salt and low Temperature in *Arabidopsis*, *PLoS ONE*, 5 (2010) e16041.

18 Lee S K, Kim B G, Kwon T R, Jeong M J, Park S *et al*, Over-expression of the mitogen-activated protein kinase gene OsMAPK33 enhances sensitivity to salt stress in rice, *J Biosci*, 36 (2011) 139-151.

19 Ravindran P N, Nirmalbabu K, Sasikumar S & Krishnamurthy K S, Botany and crop improvement of black pepper in black pepper, *Piper nigrum* edited by Ravindran P N, Harwood publishers, Singapore, (2000) 23-142.

20 Vanaja T, Neema V P, Mammootty K P & Rajeshkumar R, Development of a promising interspecific hybrid in black pepper (*Piper nigrum* L.) for *Phytophthora* foot rot resistance, *Euphytica*, 161 (2008) 437-445.

21 Dicto J & Manjula S, Identification of *PR-5* gene homologue in *Piper colubrinum* Link by suppression subtractive hybridization, *Curr Sci*, 88 (2005) 624-627.

22 Mani T & Manjula S, Optimization of *Agrobacterium*-mediated transient gene expression and endogenous gene silencing in *Piper colubrinum* Link. by vacuum infiltration, *Plant Cell Tiss Organ Cult*, 105 (2011) 113-119.

23 Krishnan A, Mahadevan C, Mani T & Sakuntala M, Virus-induced gene silencing (VIGS) for elucidation of pathogen defense role of serine/threonine protein kinase in the non-model plant *Piper colubrinum* Link, *Plant Cell Tissue Org Cult*, 122 (2015) 269-283.

24 Cao A, Xing L, Wang X, Yang X, Wang W *et al*, Serine/threonine kinase gene *Stpk-V*, a key member of powdery mildew resistance gene *Pm21*, confers powdery mildew resistance in wheat, Proc Natl Acad Sci USA, 108 (2011) 7727-7732.

25 Eulgem T, Regulation of the *Arabidopsis* defense transcriptome, *Trends Plant Sci*, 10 (2005) 71-78.

26 Martin G B, Brommonschenkel S H, Chunwongse J, Frary A, Ganal M W *et al*, Map-based cloning of a protein kinase gene conferring disease resistance in tomato, *Science*, 262 (1993) 1432-1436.

27 Song W Y, Wang G L, Chen L L, Kim H S, Pi L Y *et al*, A receptor kinase-like protein encoded by the rice disease resistance gene, *Xa*21, *Science*, 270 (1995) 1804-1806.

28 Ramonell K M & Somerville S, The genomics parade of defense responses: To infinity and beyond, *Curr Opin Plant Biol*, 5 (2002) 291-294.

29 Sheen J, Discover and connect cellular signaling, *Plant Physiol*, 154 (2010) 562-566.

30 Bent A F & Mackey D, Elicitors, effectors, and r genes: The new paradigm and a lifetime supply of questions, *Ann Rev Phytopath*, 45 (2007) 399-436.

31 Kim D S & Hwang B K, The pepper receptor-like cytoplasmic protein kinase CaPIK1 is involved in plant signaling of defense and cell-death responses, *The Plant Journal*, 66 (2010) 642-655.

32 Liu J, Elmore J M, Lin Z J D & Coaker G, A Receptor-like cytoplasmic kinase phosphorylates the host target RIN4, leading to the activation of a plant innate immune receptor, *Cell Host & Microbe*, 9 (2011) 137-146.

33 Malnoy M, Xu M, Borejsza-Wysocka E, Korban S S & Aldwinckle H S, Two receptor-like genes, *Vfa1* and *Vfa2*, confer resistance to the fungal pathogen *Venturia inaequalis* inciting apple scab disease, *Mol Plant Microb Int*, 21 (2008) 448-458.

34 Vij S, Giri J, Dansana P K, Kapoor S & Tyagi A K, The receptor-like cytoplasmic kinase (OsRLCK) gene family in rice: Organization, phylogenetic relationship, and expression during development and stress, *Mol Plant*, 1 (2008) 732-750.

35 Gao L L & Xue H W, Global analysis of expression profiles of rice receptor-like kinase genes, *Mol Plant*, 5 (2012) 143-153.

36 Chail C, Lin Y, Shen D, Wu Y, Li H *et al*, Identification and functional characterization of the soybean GmaPPO12 promoter conferring *Phytophthora sojae* induced expression, *PLoS One*, 8 (2013) e67670.

37 Kaur A, Pati P K, Pati A M, Nagpal A K, *In silico* analysis of *cis*-regulatory elements of pathogenesis-related proteins of

Aradbiopsis thaliana and *Oryza sativa*, *PLos One*, 12 (2017) e0184523.

38 Xue M, Long Y, Zhao Z, Huang G, Huang K *et al*, Isolation and characterization of a green–tissue promoter from common wild rice (*Oryza rufipogon* Griff.), *Int J Mol Sci*, 19 (2018) 1-13

39 Huda K M K, Banu M S A, Pathi K M & Tueja N, Reproductive organ and vascular specific promoter of the rice plasma membrane Ca2+ ATPase mediates environmental stress responses in plants, *PLoS ONE*, 8 (2013) e57803.

40 Bi Z, Li X, Huang H, Hua Y, Identification, functional study and promoter analysis of HbMFT1, a homolog of MFT from rubber tree (*Hevea brasiliensis*), *Int J Mol Sci*,17 (2016) 247.

41 Ayub S, Hayat R, Zainab Z, Akhtar W & Mahmodd T, OsRGLP2 promoter derived GUS expression in transgenic tobacco in response to salicylic acid, H_2O_2, PEG, NaCl and auxins, *Plant Gene*,19 (2019) 100190.

42 Zhang N, McHale & Finer J J, Changes to the core and flanking sequences of G-box elements lead to increases and decreases in gene expression in both native and synthetic soybean promoters, *Plant Biotech J*, 17 (2019) 724-735.

43 Kim J, Liu L, Hu Z & Jin E S, Identification and functional analysis of the psaD promoter of *Chlorella vulgaris* using heterologous model strains, *Int J Mol Sci*, 19 (2018) 1-14.

44 Shahan R, Li, Liu Z, Identification of genes preferentially expressed in wild strawberry receptacle fruit and demonstration of their promoter activities, *Hortic Res*, 6, 50 (2019) doi.org/10.1038/s41438-019-0134-6

45 Guo P, Zheng Y, Chen J, Wang B, Liu L *et al*, Isolation of two novel promoters from ramie (*Boehmeria nivea* L. Gaudich) and its functional characterization in *Arabidopsis thaliana*, *Plant Cell Tiss Organ Cult*,136 (2019) 467-478.

46 Bhat A, Mishra S, Kaul S & Dhar M J, Elucidation and functional characterization of CsPSY and CsUGT promoter in *Crocus sativus* L, *PLoS One*, 13 (2018) e0195348.

47 Hou J, Jiang P, Qi S, Zhang K, He Q *et al*, Isolation and functional validation of salinity and osmotic stress inducible promoter from the maize type II H+ pyrophosphatase gene by deletion analysis in transgenic tobacco plants, *Plos One*, 11 (2016) e0154041.

48 Wang C, Gao G, Cao S, Xie Q, Qi H, Isolation and functional validation of CmLOX08 promoter associated with signalling molecule and abiotic stress responses in oriental melon, *Cucumis melo* var *makuwa* Makino, *BMC Plant Biology*, 19:75 (2019) 1-14.

49 Li Q L, Xie J H, Ma X Q & Dan Li, Molecular cloning of phosphoethanolamine *N*-methyltransferase (PEAMT) gene and its promoter from the halophyte *Suaeda liaotungensis* and their response to salt stress, *Acta Physiol Plant*, 38:39 (2016) 1-8.

50 Bajaj S & Mohanty A, Recent advances in rice biotechnology-towards genetically superior transgenic rice, *Plant Biotech J*, 3 (2005) 275-307.

51 Gurr S J & Rushton P J, Engineering plants with increased disease resistance: How are we going to express it? *Trends Biotech*, 23 (2005) 283-290.

52 Kathuria H, Giri J, Tyagi H & Tyagi A K, Advances in transgenic rice biotechnology, *Crit Rev Plant Sci*, 26 (2007) 65-103.

53 Collinge D B, Jørgensen H J L, Lund O S & Lyngkjær M F, Engineering pathogen resistance in crop plants: current trends and future prospects, *Ann Rev Phytopathol*, 48 (2010) 269-91.

54 Kovalchuk N, Li M, Wittek F, Reid N, Singh R *et al*, Defensin promoters as potential tools for engineering disease resistance in cereal grains, *Plant Biotech J*, 8 (2010) 47-64.

55 Li Z, Li B & Tong Y, The contribution of distant hybridization with decaploid *Agropyron elongatum* to wheat improvement in China, *J Genet Genomics*, 35 (2008) 451-456.